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PHYSICOCHEMICAL STUDIES ON CITRATE OXALOACETATE LYASE FROM *AEROBACTER AEROGENES*

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SUMMARY

1. Citrate–oxaloacetate lyase (EC 4.1.3.6) from *Aerobacter aerogenes* has been prepared. It was homogeneous when examined in the analytical ultracentrifuge and by free boundary electrophoresis.

2. The effects of temperature, protein concentration, cysteine and urea on the stability of the preparation have been studied.

3. The diffusion coefficient was found to be $4.16 \cdot 10^{-7}$ cm^{2}sec^{-1} in the range of protein concentration from 4.3–6.4 mg/ml.

4. A value of $s^\circ_{20,w} = 16.2$ was obtained for the sedimentation coefficient.

5. A molecular weight of 318 000 was obtained by the sedimentation diffusion method. Approach-to-equilibrium studies led to a TRAUTMAN plot which gave a molecular weight of 314 000.

INTRODUCTION

Citrate–oxaloacetate lyase (citrate lyase, EC 4.1.3.6) has previously been known as citridesmolase, citrase, citratase or citrate aldolase. It is an enzyme that catalyses the breakdown of citrate to oxaloacetate and acetate and requires the presence of divalent metal ions such as Mg^{2+}. It may be induced in *Aerobacter aerogenes*[1,2], in *Streptococcus faecalis*[3,4], or in *Escherichia coli*[5,6]. The enzyme has been obtained in highly purified condition from *E. coli* by BOWEN AND SIVA RAMAN[7], and from *A. aerogenes* by SIVA RAMAN[8]. The present work is concerned with the physical properties of the enzyme from *A. aerogenes*.

MATERIALS AND METHODS

Enzyme

Citrate lyase was prepared as described by SIVA RAMAN[8] but with the omission of the first alumina C-γ-gel treatment. Table I summarises the results of a typical purification while Fig. 1 shows the Schlieren patterns of the crude starting material and the purified enzyme when run under similar conditions in the ultracentrifuge. The crude extract which was the starting material was an extract in 0.03 M KH_2PO_4

TABLE I

SUMMARY OF TYPICAL PURIFICATION OF CITRATE LYASE FROM *A. aerogenes*
Full details of method used are given by SIVA RAMAN[9].

Stage in purification	*Volume (ml)*	*Protein (mg/ml)*	*Specific activity (μmole keto acid/min/mg protein)*	*Recovery of activity (%)*
Crude extract	150	6.4	1.25	100
Streptomycin treatment	150	3.7	1.93	90
Alumina C-γ gel and $(NH_4)_2SO_4$ fractionation	40	1.85	11.5	65
Dialysis of concentrate	2	9.0	23	35
Chromatography and concentration	1	6.7	46	27

brought to pH 7 by the addition of 5 M NaOH. This extract was prepared from the crushed bacterial cells obtained by means of a Hughes' press and had been clarified at 15 000 × *g* for 20 min in the 8 × 50 ml rotor of an M.S.E. centrifuge. The crude extract then contained approx. 5 mg of protein per ml. In the final stage of the purification the enzyme was eluted from a column packed with DEAE-cellulose over the approximate range 0.15–0.25 M KH_2PO_4, brought to pH 7.4 by the addition of 5 M NaOH and containing 1.6 mM $MgSO_4$. The eluate contained 7 mg of protein in 40 ml and this was concentrated to 7 mg/ml by precipitation at half saturation with ammonium sulphate and re-dissolving in the buffer chosen for the subsequent physical determinations.

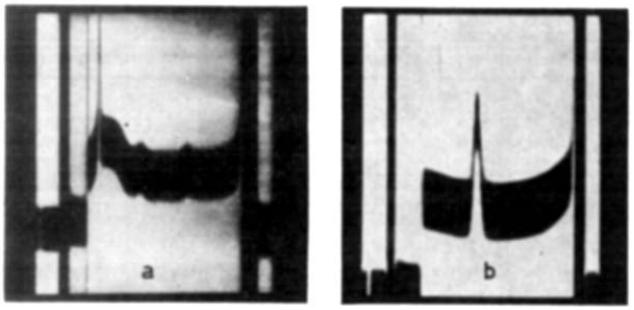

Fig. 1. Comparison of crude extract and purified enzyme in the ultracentrifuge. Both pictures were taken after 40 min at 50 740 rev./min. Bar angles 50° (a) and 40° (b). Crude extract (a) had a protein concentration of 5 mg/ml and purified citrate lyase (b) was at 3 mg/ml.

The activity of the enzyme was determined by incubation of extracts at 37° with a solution containing 20 g/l of trisodium citrate dihydrate and 5 g/l KH_2PO_4 buffered to pH 7 with 5 M NaOH. This citrate test medium also contained 1.6 mM $MgSO_4$. Protein was precipitated by the addition of an equal volume of cold 10% w/w trichloroacetic acid and the keto acid concentration in the clear supernatant was determined by the method of FRIEDEMANN AND HAUGEN[9].

Protein estimations were made by the method of FOLIN AND CIOCALTEU[10] as modified by LOWRY, ROSEBROUGH, FARR AND RANDALL[11] using crystallized bovine plasma albumin as a reference protein.

Unless otherwise stated, all measurements were carried out on the enzyme when dissolved in 0.03 M KH_2PO_4 brought to pH 7.0 by the addition of 5 M NaOH and containing 1.6 mM $MgSO_4$.

Stability

The stability of the enzyme when stored under various conditions needs special mention since a fall in activity can be correlated with a decrease in the area of the enzyme peak seen in the ultracentrifuge and the appearance of a slower moving peak with a sedimentation coefficient of approx. 12 S (Fig. 2). Extensive manipulation of the enzyme also gave rise to this slower peak and in some preparations a small amount of this component was found. The best preparations were free from the 12-S component and preparations containing more than a few per cent of it were not used.

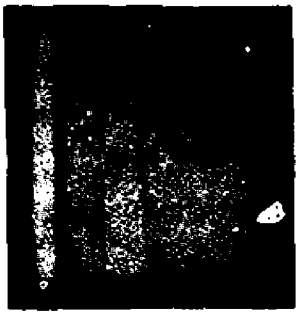

Fig. 2. Ultracentrifuge Schlieren photograph showing presence of 12 S material on extensive degradation of citrate lyase. Protein concentration 5.5 mg/ml. Picture taken after 32 min at 50 740 rev./min. Bar angle 40°.

At 20° activity fell by 20% in 5.5 h for a solution containing 3 mg/ml of protein but by only 5% over the same time at 4°. Cysteine did not stabilize the enzyme. The purified material was destroyed rapidly at 55° but the activity of the enzyme in the crude extract was little changed after 10 min at this temperature. Dilution of the pure enzyme caused a rapid loss of activity and at 20° a solution containing 0.4 mg/ml of protein lost all its activity in 2 h.

Storage at −14° was accompanied by loss of activity and a solution containing 3 mg/ml of protein lost half its activity after 4 weeks. Weaker solutions decayed more rapidly. On storage in the frozen state there was initially a rise in activity before the later decay. This effect was also observed when crude preparations were stored.

Treatment with 5 M urea at pH 6.5–7.0 caused extensive degradation of the enzyme to products with very low sedimentation coefficients; these products were not identical with the 12-S material mentioned above. 2 M urea caused no breakdown of the enzyme in 20 min.

Electrophoresis

Electrophoresis was carried out in the Kern apparatus[12,13] (Kern and Co., Aarau, Switzerland). Mobilities were not determined but were fairly low. After

1.25 h at 4.3 V/cm only a single symmetrical boundary was obtained in a solution containing 0.15 M NaCl added to the buffer mentioned above. A similar result was observed after 1 h at 5.8 V/cm in 0.1 M NaH_2PO_4 solution containing 1.6 mM $MgSO_4$ brought to pH 6.0 with the addition of 5 M NaOH. In both runs the single peak accounted for over 95% of the protein in the cell.

Sedimentation

Studies on sedimentation were carried out in a Spinco Model E ultracentrifuge equipped with rotor-temperature indication and control facilities. Phase plate Schlieren optics were available for the later stages of the work which were more particularly concerned with determination of the molecular weight. In each experiment rotor speeds given by odometer readings agreed with set values to within 0.1%. At rest the distance from the outer edge of the inner index hole to the centre of rotation was found by direct measurement to be 5.714 cm which agreed with the value give by BALDWIN[14]. Rotor stretching at speed was determined as described by KEGELES AND GUTTER[15] and was 0.29 mm at 50 740 rev./min.

The dial reading of the rotor-temperature indication and control unit was calibrated in the usual manner with the rotor at rest. A check was performed by the diphenyl ether method with the rotor spinning[16] and at 29 400 rev./min the calibration was 0.92° higher than the rest value and at 59 780 rev./min it was 1.16° higher. This discrepancy was reported and discussed by BALDWIN[14] and his example has been followed here by using the rest value.

A two-dimensional travelling microscope (Precision Grinding Ltd., Mitcham Junction Surrey, Great Britain) fitted with a projection attachment was used for all measurements on the photographic plates. ARCHIBALD runs[17,18] were usually carried out at 8900 rev./min and at this speed, and in the buffers used, there was no difficulty experienced in respect of curvature of the base line. All determinations were made at the meniscus since a reliable extrapolation of the refractive-index gradient could not be obtained at the cell bottom. CCl_4 was used in an attempt to improve the extrapolation at the cell bottom but it was found that the enzyme became denatured by this treatment. The error in the extrapolation for the gradient at the meniscus was reduced by running the solutions at such speeds and times that the Schlieren curve flattens out at the meniscus, as suggested by EHRENBERG[18]. On analysis of the Schlieren patterns measurements were started in the centre of the cell and 5 or 6 points were employed to establish the plateau region. At the boundary position some 20 points were taken at intervals of 0.05 or 0.10 mm over the linear portion of the Schlieren pattern. These points were plotted on graph paper and the best line drawn through the points to the meniscus position. Values from the line were taken at suitable intervals to give points for subsequent calculation in the manner described by KIELEY AND HARRINGTON[19]. By a plot of $(dc/dx)/xc$ *versus* x(cm)[17,20,21], where c is the increment to the index of refraction of the solution contributed by the solute component, it was found that no correction was necessary for the location of meniscus position. A synthetic boundary cell was used to obtain the proportionality constant between concentration and area of Schlieren peak.

Sedimentation coefficients were obtained usually at 50 740 rev./min but the values obtained were independent of rotor speed. Most of the ultracentrifuge runs

were carried out in the phosphate buffer previously described, but the addition of 0.15 M NaCl to the buffer did not affect the measurements which were all made at 20°.

Diffusion

Diffusion coefficients were obtained in the ultracentrifuge at 20° (refs. 22, 23). A synthetic boundary cell was used to form the sharp interface between buffer and solution. Phase-plate Schlieren photographs were taken of the diffusing boundary and the diffusion coefficients were calculated by the area–maximum height method. A zero-time correction was applied as described by SVENSSON AND THOMPSON[24]. At rotor speeds of 5102 rev./min it was possible to neglect broadening due to the dependence of sedimentation on concentration. Practical difficulties in the formation of stable boundaries prevented the use of runs on protein concentrations much below 4.3 mg/ml.

RESULTS

Fig. 3 shows the variation of the sedimentation coefficient with concentration; an extrapolated value for $s^{\circ}_{20,w}$ of 16.2 was taken. The diffusion coefficients obtained

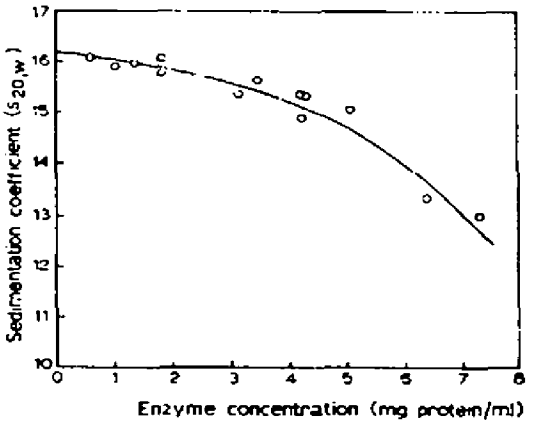

Fig. 3. Concentration dependence of the sedimentation coefficient of citrate lyase.

are shown in Table II. Since they showed no obvious trend with concentration an average value of $D_{20,w}$ of $4.16 \cdot 10^{-7}$ cm^2 sec^{-1} was taken. It was earlier pointed out that rapid decay set in when the enzyme was diluted and that material sedimenting at 12 S appeared. For this reason D values obtained at low concentrations might not refer to the pure enzyme. A partial specific volume of 0.73 was assumed as indicated by KRAGH[25]. Application of the sedimentation–diffusion equation to the results when using s values at the same concentration range as the D values gave a molecular weight of 318 000.

The ARCHIBALD method[17,26] was also used to determine the molecular weight and a TRAUTMAN plot[21] obtained as described by JONES, BABCOCK, TAYLOR AND SENTI[26]. The TRAUTMAN plot[21] (Fig. 4) included the results from 6 runs at different concentrations in the range from 2.60–7.43 mg/ml of protein. The data have been

TABLE II

VALUES OF THE DIFFUSION COEFFICIENT OF CITRATE LYASE

Protein concentration (mg/ml)	4.3	4.3	5.5	5.5	6.4
$D_{20,w}$ *(cm²sec⁻¹ × 10⁷)*	4.26	3.99	4.27	4.04	4.23

normalised to the higher figure in the range of concentration quoted. The points lay very close to a straight line so that the molecular weight was independent of concentration over the range studied and the enzyme was homogeneous. The slope of the plot gave a molecular weight value of 314 000. The average of 13 separate determinations at different concentrations within the above range gave an average molecular weight of 314 000 with a standard deviation of ± 13 800.

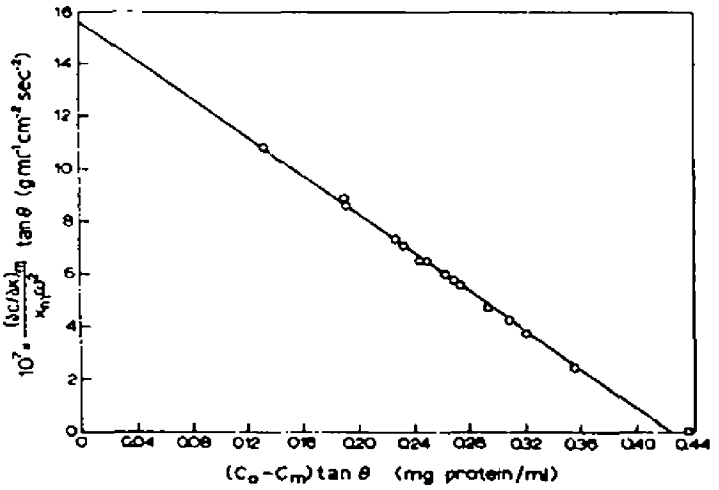

Fig. 4. TRAUTMAN plot of approach-to-equilibrium data for citrate lyase. Data normalised as described in text.

As a check on the accuracy of the methods used a sample of crystallized bovine plasma albumin was used without any special precautions to check its purity. Only one experiment was performed giving a value of $D_{20,w} = 6.45 \cdot 10^{-7}$ cm²sec⁻¹ at a protein concentration of 5 mg/ml and a molecular weight by the ARCHIBALD method[17,29] of 66 000 at protein concentration 14 mg/ml. These values were in reasonable agreement with values in the literature[14,27-29].

The form of the concentration dependence of s and the values obtained for the molecular weight and diffusion coefficient would appear to suggest that citrate lyase has a compact and possibly near spherical shape.

DISCUSSION

The occurrence of a component in crude bacterial extracts running at 12–13 S in the ultracentrifuge was first reported by BOWEN AND DAGLEY[30] and was shown to

arise when citrate lyase was induced. It was later found that various adaptive enzymes ran very close to this position in the ultracentrifuge patterns. BOWEN AND DAGLEY[31] reported that oxaloacetate decarboxylase (EC 4.1.1.3) from *A. aerogenes* sedimented in this region, and also the glutamate and arginine decarboxylases (respectively, EC 4.1.1.15 and EC 4.1.1.19) as well as the citrate lyase from *E. coli*. DAGLEY AND SYKES[32] in a more extended study produced more refined values for the sedimentation data and also showed that β-galactosidase (EC 3.2.1.21) from *E. coli* showed similar sedimentation behaviour. A characteristic feature of the leading peaks in an ultracentrifuge pattern is that they are largely composed of the bacterial ribosomes. However, BOWEN AND SIVA RAMAN[7] purified the citrate lyase from *E. coli* and found it to be free of nucleic acids: it sedimented at 16 S as a result of the removal of the other cell components which were present when the earlier values of 12–13 S were reported. SIVA RAMAN[8] was able to purify the citrate lyase from *A. aerogenes* which was the subject of the work reported here. In this connection HU, WOLFE AND REITHEL[33] quoted a value of $s^{\circ}_{20,w} = 16.14$ for β-galactosidase from *E. coli* and a molecular weight of 747 000 but they also quoted a value of 365 000 for the enzyme from a different strain of the organism reported personally to them by WALLENFELS. It seems likely that the molecular weights of the bacterial induced enzymes may be very similar and the figure of 747 000 may be due to dimerisation. According to WRIGHT[34] the molecular weights of enzymes fall into 3 geometric series: he points out that the microsomal molecular weights are related approximately to a geometric series similar to that described for enzymes and has tentatively suggested that there might be a relationship. The molecular weight here found for citrate lyase would fit into such a series and there are indications that the other bacterial adaptive enzymes may also fit in as their molecular weights become available.

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